Identifying Novel Protein Interactors of Cytoplasmic Capping Enzyme Using Proximity-Dependent Biotinylation

Research Thesis

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Abstract

Cap homeostasis is a cyclical process of 5’ mRNA cap removal followed by re-addition of the cap (“recapping”) that affects a subset of mRNA transcripts. Uncapped targets of recapping can accumulate in the cytoplasm, and cytoplasmic recapping is all that is needed for the return of these transcripts to the actively translating pool. Cap homeostasis is a unique mechanism for post-transcriptional gene regulation, but the biological applications of cytoplasmic recapping are still not well understood. The current study aims to establish novel protein interactors of cytoplasmic capping enzyme (cCE), a core member of the protein machinery that is necessary to perform recapping. Using proximity-dependent biotin identification (BioID), interactors of cCE are biotin-tagged *in vivo* and identified by western blotting and mass spectrometry. Here, I discuss the implications of identified cCE interactors for cap homeostasis as well as introduce the possibility that cCE is involved in biological processes outside of cytoplasmic recapping.

Introduction

The central dogma of RNA biology in eukaryotes states that mRNA transcripts are produced in the nucleus and are matured through a series of step-wise modifications. These modifications include the addition of a 5’ m7G cap structure, addition of a polyA stretch of residues at the 3’ end, and splicing of introns (15). Mature mRNA then exits the nucleus by localizing to the nuclear pore complex and translocating into the cytoplasm (5). Once in the cytoplasm, mRNA is subject to translation from free or membrane bound ribosomes. The efficiency of translation of any

given mRNA depends on a variety of factors, but perhaps most importantly the maintenance of an intact 5’ cap (5). The cap protects the transcript from degradation by cytoplasmic exonuclease enzymes and recruits translation machinery necessary for normal protein expression (5). Eventually, all transcripts will be subject to de-capping and degradation, a process known as RNA turnover. This signals the end of transcript expression. It was previously thought that the loss of the 5’ mRNA cap is an important signal that an mRNA has begun the irreversible process of turnover.

In addition to stabilizing mRNAs, there is also evidence that a mature m7G cap structure is necessary to recruit multiple cap-binding complexes (CBCs) both in the nucleus and cytoplasm. The cap can recruit factors necessary for spliceosome assembly (1) on the pre-mRNA as well as 3’ end processing factors that bind at the polyA site in the nucleus (2). Both of these steps are essential to produce mature mRNA. CBC binding to transcripts is also thought to be essential for mRNA nuclear export in higher eukaryotes, in which associations between CBC proteins and nuclear export machinery at the nuclear envelope mediates export through the pore with 5’ to 3’ (3,4). Once in the cytoplasm, the cap indirectly recruits the initiator tRNA and ribosomal subunits via CBCs comprised of translation initiation factors (5). Cap formation then is critical for the first round of translation and maintenance of translation throughout the active period of the mRNA (5).

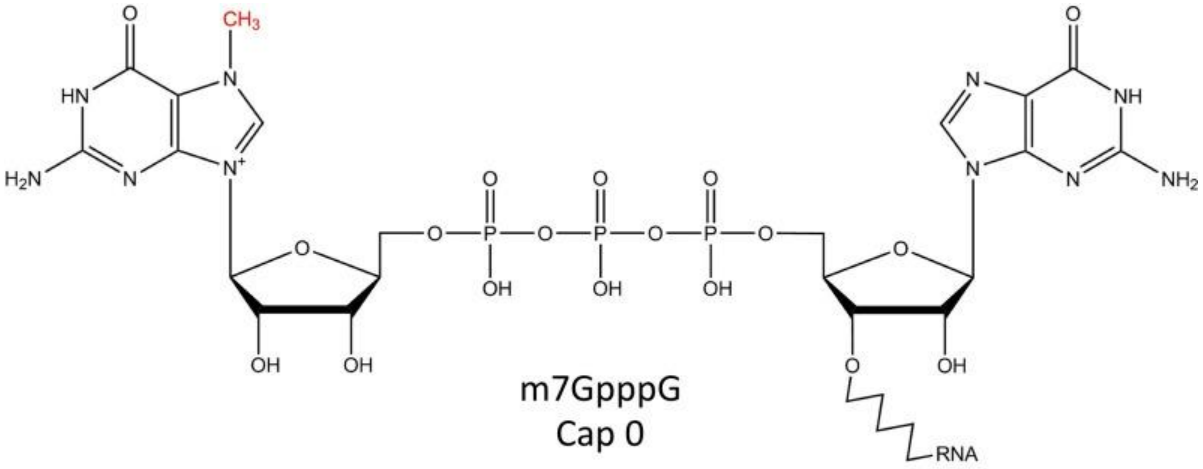

Figure 1: Eukaryotic mRNA cap consists of an inversely-oriented guanosine residue (left) that is linked to the first transcribed nucleotide (right) through a triphosphate bridge. The cap also consists of two methylations. The first methylation is at the N-7 position on the G-cap, and the second methylation is at the 2'O position on the first transcribed nucleotide. (Source: 5)

The eukaryotic mRNA cap consists of an inverted guanosine triphosphate residue that is methylated at position N7. This m7 modification on the guanosine residue is necessary to maintain a functional cap structure (11). A second methylation occurs at the +1 nucleotide at the 2'O position. This modification enables the cell to discriminate between its own transcript population and potentially harmful viral gene products, and serves as an immune defense (5). Traditionally, formation of an intact cap was thought to occur only in the nucleus via a series of stepwise modifications. These modifications include: 1) γ-phosphate cleavage of the nascent 5' triphosphate, 2) guanosine residue addition to this diphosphate intermediate, and 3) proper methylations at the N7 and 2'O positions as described above (6). The first two reactions are catalyzed by capping enzyme (CE) and the N7 methylation is catalyzed by cap-methyltransferase (RNMT). One prediction of the hypothesis that 5' capping only

occurred in the nucleus is that the identified cap-building enzymes are only functioning in the nucleus, where mRNA maturation takes place. However, an initial observation of 5' truncated forms of nonsense-containing mRNA that appeared to have a re-constituted cap structure led to the idea that there may be capping machinery in the cytoplasm (6). In addition, CE was shown to also have a cytoplasmic expression pattern (6). This, along with similar observations of cytoplasmic distributions of other capping factors, eventually led to the idea of an enzymatically active cytoplasmic capping complex (7).

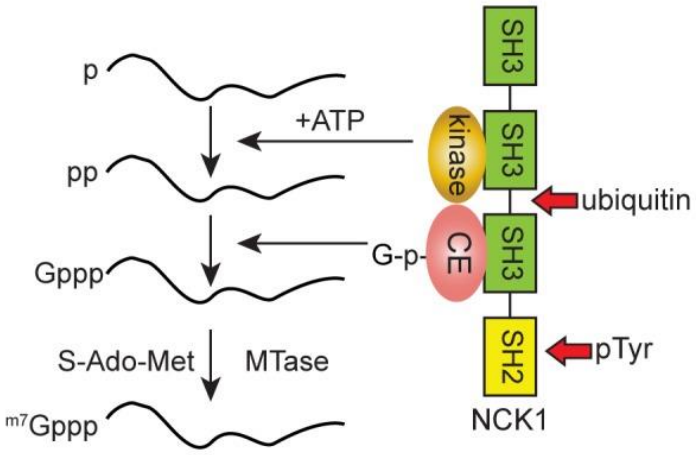

Figure 2: Early model of the cytoplasmic capping complex. An uncapped, monophosphate end is modified to a diphosphate end by an unknown cytoplasmic kinase. Capping enzyme present in the cytoplasm catalyzes addition of GMP to the phosphate end to create an unmethylated cap structure. An unknown methyltransferase (now understood to be RNMT) catalyzes m7 methylation. CE is mound via its C-terminus to Nck1, an adapter protein that also recruits monophosphate kinase activity. (Source 7)

In the cytoplasm, generation of a re-constituted cap diverges slightly from initial nuclear cap formation. The primary reaction is catalyzed by an unidentified cytoplasmic kinase which transfers the γ-phosphate of ATP to an uncapped 5' monophosphate end

of mRNA, generating a diphosphate end which can be recognized by cCE. cCE transgene expression and subsequent recovery using magnetic beads identified adapter protein Nck1's third SH3 domain as a binding partner of cCE. *In vitro* 5' kinase activity was found to be dependent on an intact second SH2 domain of Nck1 (7). These data along with evidence that cytoplasmic capping functionality is dependent on Nck1(7) led to the modern hypothesis of the cytoplasmic capping complex. Nck1 acts as a scaffold to bind the catalytic subunits of the cytoplasmic capping reactions and serves to increase re-capping efficiency by increasing the local concentration of recapping enzymes (7).

The specific biological use of cytoplasmic capping is not well understood, although it is thought to assist in cell recovery from stress (6). In general, cytoplasmic recapping is thought to restore inactive transcripts to a translationally active state, a phenomenon known as cap homeostasis (7). Cap homeostasis could provide a mechanism for the cell to express certain proteins when stressed, when translation is largely inhibited (6). Cytoplasmic recapping could also provide a fast way to respond to various internal or external stimuli. Re-activating existing cytoplasmic transcripts would allow the cell to change protein expression, and this response would be far quicker than the cell synthesizing new gene products and moving them to various subcellular locations. To this end, cytoplasmic recapping could be useful to change protein expression at a local level, since recapping can only occur where uncapped yet otherwise intact transcripts have accumulated. The intriguing potential for cytoplasmic recapping on gene regulation has motivated the current research in the Schoenberg Lab.

The goal of the current study is multifold. The first aim was to develop proximity-dependent biotinylation in the context of cytoplasmic capping as a mechanism by which to study the protein factors involved in the cytoplasmic capping process. Once established, this system was used to validate already proposed members of the core cytoplasmic capping complex, including those described above. Most importantly, this study aims to establish cCE as a potentially multifunctional protein within a vast proteome and begin to characterize its relationship to other proteins that have not yet been considered in the context of cytoplasmic capping.

The identification of protein interactors in this study uses a technique called “proximity-dependent biotin identification” (BioID) which was first developed and used by the lab of Kyle Roux in 2012 to survey protein interactors of Lamin-A, a component of the nuclear envelope (8). The utility of BioID over more traditional protein identification techniques (namely isolation of interactors via pulldown using a bait protein of interest) is that BioID can tag interacting proteins with biotin *in vivo*. While traditional methods provide a snapshot of bound proteins at any given moment, *in vivo* tagging documents a history of protein interactions over the labeling time course. The BioID approach should provide a more robust profile of protein interactors to a protein of interest than a parallel traditional pulldown. This is because BioID’s ability to tag even transient interactors with lower affinity to a protein of interest can provide a mechanism for isolation that a traditional pulldown technique does not provide. Without a biotin tag that can be recognized, these loosely associated proteins would be washed away during processing of pulldown samples.

BioID uses the biotin ligase protein BirA that comes from *Escherichia coli*. Its wild-type function is to act as a repressor in the biotin biosynthesis operon (9) and its tagging ability is highly specific to a single substrate in E. coli. For the purpose of tagging mammalian proteins, the Roux lab chose a BirA mutant (R118G) termed BirA*. Unlike the extremely specific wild-type protein, BirA* "promiscuously" tags proteins in a proximity-dependent fashion and does not require the presence of a biotin acceptor tag (BAT) necessary for wild-type tagging. BirA* biotin tags proteins in a two-step process. The first step is a free ATP addition to biotin to create biotinoyl-5′-AMP (bioAMP), which is stored in the active site of BirA*. bioAMP is then added to primary amine residues of a proximal protein. The BirA* tagging radius is thought to be about 100 angstroms (13). The result is a covalent attachment of biotin onto lysine and arginine residues of proteins proximal to BirA* at any time throughout the time course of an experiment. Additionally, the efficient conversion of biotin to bioAMP requires a higher-than endogenous level of biotin, so free biotin must be added to mammalian cell cultures.

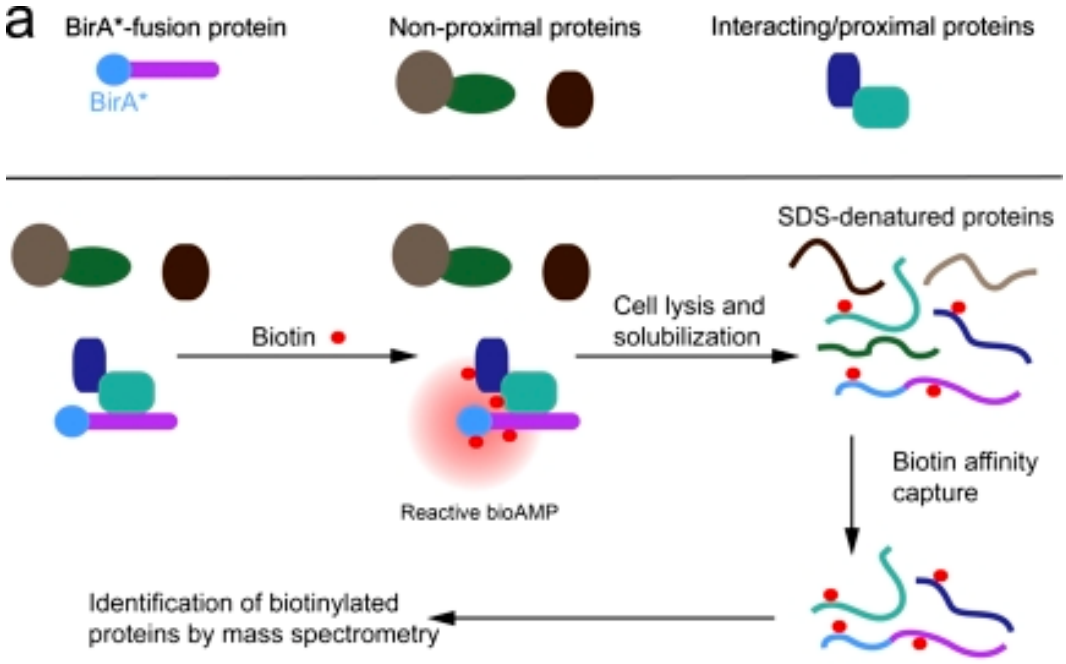

Figure 3: Proximity-dependent biotinylation with BirA fusion protein. A construct expressing the BirA* biotin ligase fused to a protein of interest is introduced into mammalian cells. While this protein is being expressed, biotin is supplemented into the media. The BirA* protein biotin tags proteins within a 100 Å radius in vivo. Tags proteins can be isolated from cell lysate by streptavidin affinity chromatography and analyzed by western blot or mass spectrometry. (Source 8)*

BirA* becomes useful as a biotin tagging mechanism when it is fused to a protein of interest (POI). In a living cell, it is assumed that a BirA*-POI construct will have the same function as the endogenous POI. Using cCE as the protein of interest, we created fusion constructs expressing BirA*-cCE. Proteins that associate with the expressed BirA*-cCE are considered interactors of cCE. By necessity, these interactors will be drawn close to BirA* during their association with the cCE. During this interaction time frame, BirA* can opportunistically label cCE-associating proteins, serving as a way to tag and later isolate associating proteins. Basal and off target biotin tagging events can

be controlled for by expressing an empty BirA* construct and introducing the same level of biotin.

Materials and Methods

Plasmid constructs

To generate pcDNA3.1-myc-BirA*-cCE, pcDNA3.1-myc-BirA* (Addgene plasmid #35700, Roux Lab) was first modified by Change-IT Site-Directed Mutagenesis using primers to change the NotI restriction site to a BsrGI restriction site, creating pcDNA3.1-myc-BirA*-BsrGI. pcDNA3.1-myc-BirA*-cCE was generated by PCR-amplifying the cCE sequence from pcDNA3.1/TO-myc-cCE-bio [4] using PCR primers and inserting into XhoI-digested pcDNA3.1-myc-BirA*- BsrGI. To generate pcDNA3- FLAG-RNMT, human RNMT with an N-terminal FLAG tag and linker sequence was PCR-amplified and inserted into KpnI-digested pcDNA3. To generate pcDNA3.1/TO-myc-BirA*-NES and pcDNA3.1/TO-myc-BirA*-cCE, the Tet-operator sequence and NES sequence (to pcDNA3.1/TO-myc-BirA* only) were inserted using Change-IT Site-Directed Mutagenesis primers. All plasmid sequences were confirmed with Sanger sequencing.

Cell Culture

Human osteosarcoma (U2OS) cells obtained from ATCC (HTB-96) were cultured in McCoy's 5A medium (Thermo Fisher 116600–108) supplemented with fetal bovine serum (FBS) to 10% (v/v). Human embryonic kidney (HEK293) cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher 11995–073) supplemented with

FBS to 10% (v/v). Cells were maintained at 37°C under 5% CO_2 and were discarded after 20 passages.

Transfection and cytoplasmic fractionation

Prior to transfection, cells were given fresh, pre-warmed medium. HEK293 or U2OS cells were 60–70% confluent at time of transfection. For the analysis of RNMT, HEK293 cells were transfected with a 10-cm dish using jetPRIME transfection reagent (Polyplus 114–15) according to manufacturer's protocol. For the analysis of Hsp90 and XPO2, U2OS cells were transfected with 6 µg plasmid DNA per 10-cm dish using FuGENE 6 transfection reagent (Promega E2691) according to manufacturer's protocol. After centrifugation at 70 × *g* for 10 min at 4°C, pellets were resuspended in approximately 10 volumes of ice-cold YO Lysis Buffer (10) (10 mM HEPES pH 7.3, 10 mM KCl, 10 mM $MgCl_2$, 0.2% (v/v) IGEPAL CA-630, 2 mM dithiothreitol (DTT), 0.5 mM PMSF, 7.5 µl/ml protease inhibitor cocktail (Sigma P8340), 7.5 µl/ml phosphatase inhibitor cocktail 2 (Sigma P5726), 7.5 µl/ml phosphatase inhibitor cocktail 3 (Sigma P0044)) by pipetting 10 times. Resuspended cells were incubated on ice for 10 min, pipetted five times, and centrifuged at 16,100 × *g* for 10 min at 4°C. The supernatants (cytoplasmic extracts) were removed and kept on ice. Cytoplasmic protein concentration in each extract was determined by Bradford assay using Bio-Rad Protein Assay Dye Reagent (Bio-Rad 5000006) normalized against a standard curve of BSA (Fisher Scientific BP1600–100). For SDS-PAGE analysis, extracts were diluted to 75% with 4× Laemmli Sample Buffer.

SDS-PAGE and western blotting

Samples in Laemmli Sample Buffer were heated at 95 °C for 5 min and briefly centrifuged before loading Mini-PROTEAN TGX gels (Bio-Rad) and electrophoresing at 150 V in Tris-glycine-SDS running buffer. Proteins were transferred to Immobilon-FL (EMD Millipore IPFL00010) PVDF membranes at 100 V for 60 min at 4 °C. Membranes were blocked in Blocking Buffer (TBS containing 3% (w/v) BSA) for at least 30 min at room temperature before incubating overnight at 4 °C in Blocking Buffer containing primary antibodies at the dilutions listed in the Antibodies section. Membranes were washed three times in TBS-T at room temperature, at least 10 min per wash. Secondary antibody incubations were performed for 30 min at room temperature in Blocking Buffer, with antibody dilutions listed in the Antibodies section. Membranes were washed again with TBST as before and then imaged with a Li-Cor Odyssey infrared scanner at 700 or 800 nm as appropriate.

Antibodies

Primary antibodies used in this study are as follows: mouse anti-myc (Santa Cruz sc-40; 1:1000 for WB, 1:1000 for IF), rabbit anti-XPO2/CSE1L (Proteintech; 1:1000), rabbit anti-HSP90α/β (Santa Cruz sc-4947; 1:2000), rabbit polyclonal anti-CE (Novus NBP1-49973; 1:2000), and mouse monoclonal anti-FLAG (Sigma F3165; 1:1000). Qdot 800 streptavidin conjugate (Thermo Fisher Q10171MP; 1:10,000 for WB) was used to detect biotinylated proteins.

Streptavidin pulldown

After 24 h induction with tetracycline-HCl, growth medium was completely removed from culture dishes. Cells were then washed with PBS and harvested by scraping into PBS. Samples prepared for mass spectrometry analysis that came from multiple dishes were pooled into a single centrifuge tube. Cytoplasmic extracts were prepared as previous described. To tubes containing 15 µL (stock slurry volume) of MyOne Streptavidin T1 Dynabeads (Thermo Fisher 65601), equal mass amounts of protein (amounts varied for WB experiments; 1.6 mg per sample used for mass spectrometry-analyzed samples) were added, brought to 500 µL with YO Lysis Buffer (Trotman et al., 2017), and brought to 150 mM upon addition of 15 µL of 5 M NaCl to constitute Pulldown Buffer containing recovered samples. For pulldowns done in Pulldown Buffer+SDS, 5 µL 10% (w/v) SDS was added to each 500 µL mixture to create a final concentration of 0.01% SDS per pulldown reaction. Tubes were incubated end-over-end for 1 h at 4 °C to bind biotinylated proteins to the beads. The beads were washed 3 times with 500 µL of ice-cold YO Lysis Buffer containing 150 mM NaCl, incubating end-over-end at 4 °C for 10 min per wash. Mass spectrometry-analyzed samples were then washed 3 times with 500 µL of ice-cold 50 mM ammonium bicarbonate, incubating end-over-end at 4 °C for 10 min per wash. After removal of the final wash, beads were resuspended in either 20 µL 50 mM ammonium bicarbonate (for proteomics analysis) or 15-50 µL 2× Laemmli Sample Buffer (Bio-Rad 1610737) containing 5% (v/v) β-mercaptoethanol. Samples were stored at -20 °C or immediately analyzed by western blot.

RNMT analysis by transient transfection

Triplicate cultures of HEK293 cells were transiently transfected with a 1:3 (w/w) ratio of pcDNA3-FLAG-RNMT and either pcDNA3.1-myc-BirA* or pcDNA3.1-myc-BirA*-cCE. The total amount of transfected DNA was 10 µg. After 6 h, a stock solution of 10 mM biotin (Sigma-Aldrich B4639–1G) in dimethylsulfoxide was prepared and added directly to medium to final concentrations of 5 and 25 µM biotin, or DMSO without biotin as a control. Additional DMSO was added where necessary to normalize the volume of mix added to each dish. Cells were incubated 14 h before harvesting and preparing cytoplasmic extracts as described above. Streptavidin pulldown was performed as described above, using 300 µg total cytoplasmic protein. Beads were resuspended in Laemmli Sample Buffer and analyzed by SDS-PAGE and western blot. Using the same cytoplasmic extracts, anti-FLAG immunoprecipitation was performed as described below.

FLAG Immunoprecipitation

Quantified cytoplasmic extracts (1000 µg each) were brought to 500 µl with Combo Buffer and pre-cleared with 15 µl slurry-equivalent of Dynabeads Protein G (Thermo Fisher 10003D) at 4°C for 45 min with end-over-end rotation. At the same time, 15 µl slurry-equivalent Dynabeads Protein G was incubated with 2 µg FLAG antibody in 500 µl Combo Buffer (for each immunoprecipitation) at 25°C for 45 min. Pre-cleared extract samples were then incubated with bead-bound antibodies end-over-end at 4°C overnight. Beads were washed three times with 500 µl Combo Buffer, 10 min end-over-

end at 4°C per wash. Beads were resuspended in to 2× Laemmli Sample Buffer for SDS-PAGE.

Hsp90 and Exportin 2 analysis by transient transfection

HEK293 cells were transiently transfected with either pcDNA3.1/TO-myc-BirA*-NES or pcDNA3.1/TO-myc-BirA*-cCE. The total amount of transfected DNA was 6 µg. After 6 h, 1µM biotin was added directly to the medium. Cells were incubated 14 h before harvesting and preparing cytoplasmic extracts as described above. Streptavidin pulldown was performed as described above, using 1000 µg total cytoplasmic protein. Beads were resuspended in Laemmli Sample Buffer and analyzed by SDS-PAGE and western blot. An additional streptavidin pulldown was performed with 0.01% SDS as described above.

Stable cell generation

To generate stable U2OS-TR cell lines expressing BirA* and BirA*-cCE, U2OS-TR cells were transfected with either pcDNA3.1/TO-myc-BirA*-NES or pcDNA3.1/TO-myc-BirA*-cCE using FuGene 6 (Promega E2691) according to manufacturer's instructions. Cells were passaged two days following transfection and then maintained for two weeks in medium containing 600 µg/mL Geneticin (Thermo Fisher 10131035). Maintaining selective pressure with 600 µg/mL Geneticin, BirA* cells were passaged to low densities to allow formation of individual colonies that were isolated using cloning cylinders. Cell lines were expanded over several weeks in medium containing 600 µg/mL Geneticin before confirming inducible expression of myc-BirA*. Myc-BirA*-cCE

were maintained in antibiotic as described above but were not isolated as single colonies. Inducible expression of each cell line was confirmed by western blotting and immunofluorescence as described below.

Immunofluorescence

Glass coverslips in the wells of a 24-well plate were each seeded with 50,000 stable cells 17 h prior to induction. Expression of BirA* was induced by addition of 0.02 µg/mL tetracycline-HCl BirA*-cCE was induced by addition of 0.2 µg/mL tetracycline-HCl. After 24 h, medium was removed from the coverslips, and the cells were fixed for 20 min at room temperature in PBS containing 4% formaldehyde and 0.2% (v/v) Triton X-100. Coverslips were briefly washed three times with PBS before incubating in IF Block Solution (PBS containing 1% (w/v) BSA and 0.05% (v/v) Triton X-100) for 90 min at room temperature, followed by overnight incubation at 4 °C in IF Blocking Solution containing a 1:1000 dilution of mouse anti-myc antibody (Santa Cruz sc-40). Coverslips were washed three times with IF Wash Solution (PBS containing 0.5 mM $MgCl_2$ and 0.05% (v/v) Triton X-100) at room temp, 5 min per wash, and then incubated for 60 min in the dark at room temp in IF Block Solution containing 0.75 µg/mL DAPI and a 1:1000 dilution of anti-mouse Alexa Fluor 594 (Thermo Fisher A11005). After washing with IF Wash Solution as before, coverslips were mounted on glass microscope slides with ProLong Gold Antifade Mountant (Thermo Fisher P36930), and incubated overnight in the dark at room temp to allow the mountant to cure. Images were acquired at room temp with a Nikon Eclipse Ti-U inverted microscope fitted with a CFI Plan Apo VC 60X oil immersion objective and a Nikon DS-Qi1 monochrome digital camera. Images were

analyzed using Nikon NIS-Elements AR 3.10 software. Specificity of the secondary antibody for the primary antibodies was confirmed by parallel preparation of control coverslips not treated with primary antibody.

Proteomics analysis

Beads from streptavidin pulldowns were reconstituted in 80 µL of 50 mM ammonium bicarbonate containing 0.02% ProteaseMAX surfactant (Promega V2071) and incubated at 95 °C for 5 min. After adding 13 µL of 50 mM ammonium bicarbonate, 1 µL of 0.5 M DTT was added, and samples were incubated at 56 °C for 20 min for the reduction of disulfides. Cysteine alkylation of was then performed by adding 3 µL of 0.55 M iodoacetamide and incubating at room temp in the dark for 15 min. Tryptic digests were performed by adding 1 µL of 1% ProteaseMAX surfactant and 2 µL of 1 µg/µL trypsin and incubating at 37 °C for 3 h. Trypsin was inactivated upon addition of trifluoroacetic acid (TFA) to a final concentration of 0.5%. The digestion products were centrifuged 16,000 × *g* for 10 min, and the supernatants were cleaned up with C18 cartridge columns. Approximately 0.5 µg of peptides per sample were separated by reversed-phase ultra-performance liquid chromatography (RP-UPLC) on a Waters nanoACQUITY UPLC system and mass analyzed by a ThermoFisher Orbitrap Elite using top 15 data dependent acquisition in positive ion mode.

Results

BioID confirms known cytoplasmic capping complex members.

BirA* and Myc tags were added to the N-terminus of a form of CE that is restricted to the cytoplasm due to loss of the NLS (nuclear localization sequence) and addition of the HIV Rev NES nuclear export sequence) to create myc-BirA*-cCE. These modifications to CE were originally shown to be sufficient to restrict CE almost completely to the cytoplasm (6). A parallel construct expressing myc-BirA* without CE was used as a control for non-specific biotinylation.

These constructs were first used to determine if there was a proximal relationship between cCE and RNMT (cap-methyltransferase). RNMT protein was previously shown to be expressed in the cytoplasm of U2OS cells (11). Initially, an approach was taken to preferentially recover endogenous RNMT from cytoplasmic extracts in the presence of BirA*-cCE. But, endogenous RNMT was not detected by western blot. This may have been due to the low level of cytoplasmic RNMT compared to other members of the cytoplasmic capping complex. We then moved to a co-transfection approach in which we overexpressed a tagged form of RNMT together with a BirA* construct. Plasmids expressing either myc-BirA* or myc-BirA*-cCE were transfected into HEK293 cells together with a second plasmid expressing FLAG-RNMT and incubated for 6 h. Cells were then cultured for 14 h in the same medium supplemented 0, 5 or 25 μM biotin. Cytoplasmic extracts recovered on streptavidin beads were then analyzed by western blotting using antibodies against the FLAG tag, Nck1, and RAM.

Cytoplasmic BirA* and BirA*-cCE were expressed regardless of biotin supplementation (Figure 4A, left panel), and their recovery on streptavidin beads shows that biotinylation events are dependent on biotin supplementation (Figure 4A, right panel). The recovery of Nck1 from only from cells expressing BirA*-cCE confirmed the

utility of BioID for identifying known cytoplasmic capping complex components. Selective recovery of RNMT confirmed its identification as a component of the cytoplasmic capping complex. In addition, the absence of GAPDH, a highly expressed and assumed unrelated cytoplasmic enzyme, confirmed the specific biotin labeling of proteins in the cytoplasmic capping complex. RNMT functions as a heterodimer with a 118-amino acid protein termed RNMT-activating miniprotein (RAM) (12) RAM is necessary for stabilization of RNMT in the cytoplasm but is not though to have catalytic activity related to cytoplasmic 5’ capping. RAM was present in the input cytoplasmic extract and, like Nck1 and RNMT, was selectively recovered with BirA*-cCE.

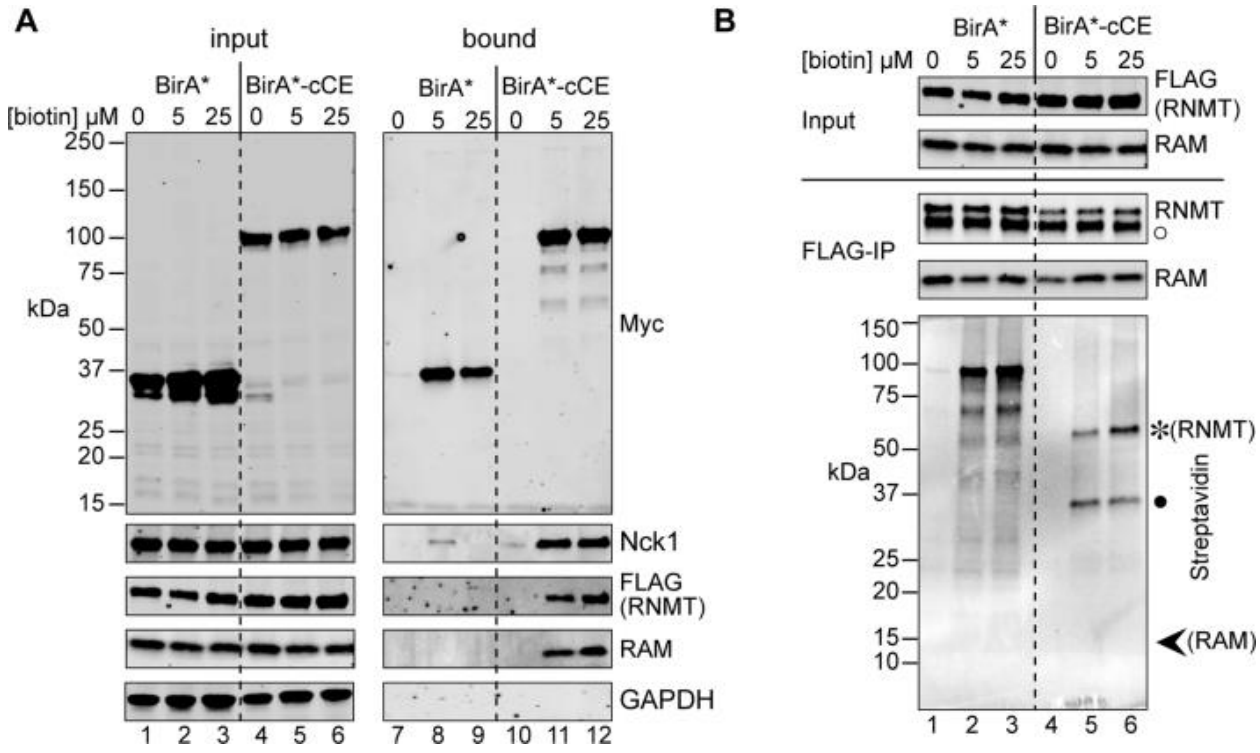

Figure 4: BioID identifies Nck1, RNMT, and RAM as members of the cytoplasmic capping complex. A: HEK293 cells were cotransfected with plasmids expressing either Myc-BirA or Myc-BirA*-cCE together with FLAG-RNMT. After transfection, cells were supplemented with either 5 or 25 µM biotin in DMSO, or with only DMSO vehicle as a control. Cytoplasmic extracts were isolated and biotinylated proteins were recovered on streptavidin beads. Recovered samples were analyzed by western blotting with antibodies against Myc (to visualize BirA* and BirA*-cCE), Nck1, FLAG (to visualize FLAG-RNMT), RAM, and GAPDH. B: The same cytoplasmic extracts were immunoprecipitated using the antibody against the FLAG tag used in (A). IP samples were analyzed by western blot with antibodies against RNMT and RAM to confirm their recovery. The open circle in the RNMT panel represents the heavy chain of the FLAG antibody. The same IP samples were then probed with streptavidin to visualize biotin tagging of proteins. The sizes of RNMT, RAM, and an unknown protein denoted by a black dot are indicated. (Source 10)*

These results do not indicate if RNMT and RAM themselves were biotinylated by BirA*-cCE or if their recovery was caused by an association to some other biotinylated protein. To address this, FLAG-RNMT was recovered from the same extracts by

immunoprecipitation, and biotinylated proteins were visualized by streptavidin western blot (Figure 4B). The recovery of RAM with RNMT confirmed that these proteins interact with each other in the cytoplasm. Streptavidin western blotting showed RNMT was biotinylated by BirA*-cCE. RAM was not detected with streptavidin. This could be either to RAM being too far away from BirA* in complex to be biotinylated or because of a shielding by effect by RNMT. Regardless of the biotinylation state of recovered proteins, the propensity for RNMT and RAM to be preferentially recovered in BirA*-cCE samples suggests that they are members of a cytoplasmic capping complex. FLAG-RNMT also recovered a 30 kDa protein (solid dot) whose biotinylation by BirA*-cCE identified it as an unknown constituent of the cytoplasmic capping complex that may contribute to cap methylation. This protein has yet to be identified.

Experiments in (10) performed by Jackson Trotman using recombinant forms of tagged RNMT and tagged CE suggested that RNMT and CE can bind directly to each other *in vitro*. Jackson expanded these results using a bio-cCE construct transfected into HEK293 cells along with along with one of three forms of RNMT (either full length, N-terminal deletion, or catalytic C-terminal deletion). Streptavidin recovery of bio-cCE as a bait protein brought along both full-length RNMT and the N-terminal deletion construct. Thus, residues within the C-terminal methyltransferase domain seem to be responsible for recruitment of RNMT into the cytoplasmic capping complex. These data, along with proximity dependent biotinylation of FLAG-RNMT using BirA*-cCE indicates that RNMT is recruited as a member of the cytoplasmic capping complex via a direct association to cCE (Figure 5).

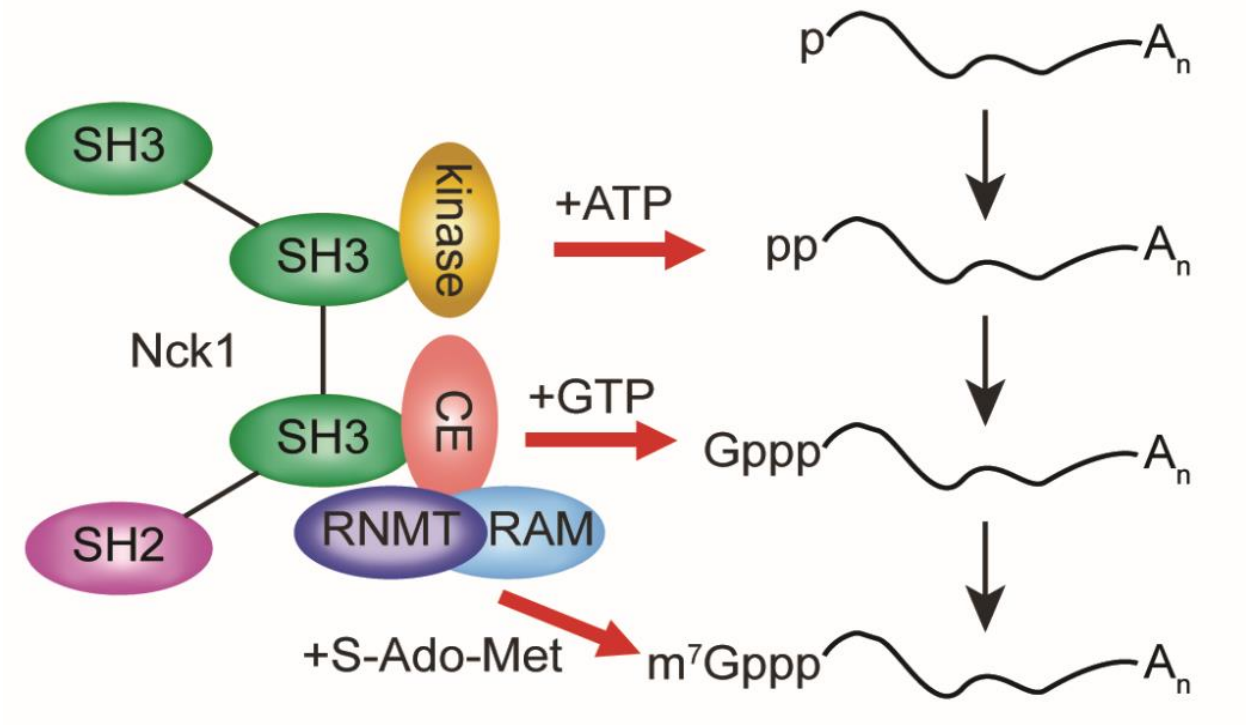

Figure 5: The core cytoplasmic capping complex. (Courtesy of Dr. Daniel Schoenberg)

BioID confirms association of ΔN-RNMT to the cytoplasmic capping complex

As part of work to determine whether RNMT was responsible for the catalysis of the N7 methylation of the cytoplasmic cap, a catalytically inactive form of RNMT was constructed by Jackson Trotman, termed ΔN-RNMT. The D203A mutation in the otherwise intact C-terminal domain disrupts S-Adenosyl methionine (SAM), the methyl group donor needed or RNMT catalysis, from binding to its binding pocket on RNMT (Figure 6A). Notably, ΔN-RNMT retains the ability to bind RAM. In order for ΔN-RNMT to be considered a dominant-negative inhibitor of cytoplasmic capping, it was necessary to establish that expressed ΔN-RNMT is capable of associating with the cytoplasmic capping complex in a manner indistinguishable to that of wild-type RNMT. This question was examined in an experiment parallel to that shown in Figure 4A, in which a co-

transfection of BirA*-cCE or BirA* and FLAG-ΔN-RNMT was supplemented with biotin before performing a streptavidin pulldown and western blot analysis. Selective recovery of ΔN-RNMT with BirA*-cCE only in during biotin supplementation (Figure 6B, right panel) mirrored the result shown with wild-type RNMT (Figure 4B, right panel) indicating that the catalytically inactive ΔN-RNMT mutant interacts with the cytoplasmic capping complex just as well as wild-type RNMT.

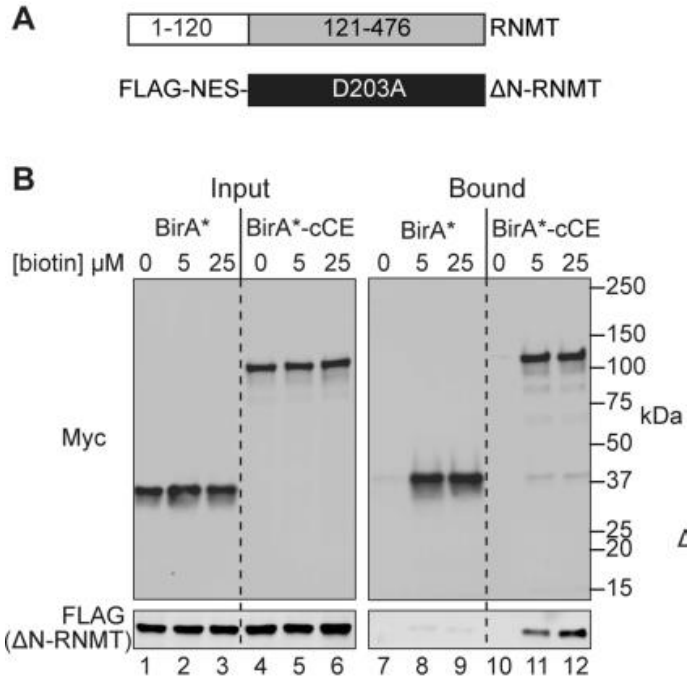

Figure 6: ΔN RNMT interacts with cCE in a manner indistinguishable to wild-type RNMT. A: A schematic of the FLAG-ΔN-RNMT construct and how it differs from the FLAG-RNMT construct used in Figure 4. B: Cells were transfected and prepared as in Figure 4A, except that FLAG-RNMT was replaced with FLAG-ΔN-RNMT. Recovered samples were analyzed with antibodies against Myc (to visualize Myc-BirA and Myc-BirA*-cCE) and FLAG (to visualize FLAG-ΔN-RNMT). (Source 10)*

Transfection of a plasmid expressing ΔN-RNMT results in a highly expressed, catalytically inactive form of RNMT that is still able to associate with members of the

cytoplasmic capping complex (Figure 6). This situation presumably results in majority of cytoplasmic capping machinery associating in complexes that include ΔN-RNMT instead of wild-RNMT. Such complexes are predicted to be catalytically incomplete, because ΔN-RNMT is unable to complete cap methylation. This is likely to result in a reduced ability to create mature caps in the cytoplasm. This hypothesis was confirmed by Jackson Trotman by using expression of ΔN-RNMT to destabilize known mRNA targets of cytoplasmic recapping and confirm that catalytically active RNMT is essential for fully-efficient re-capping (10).

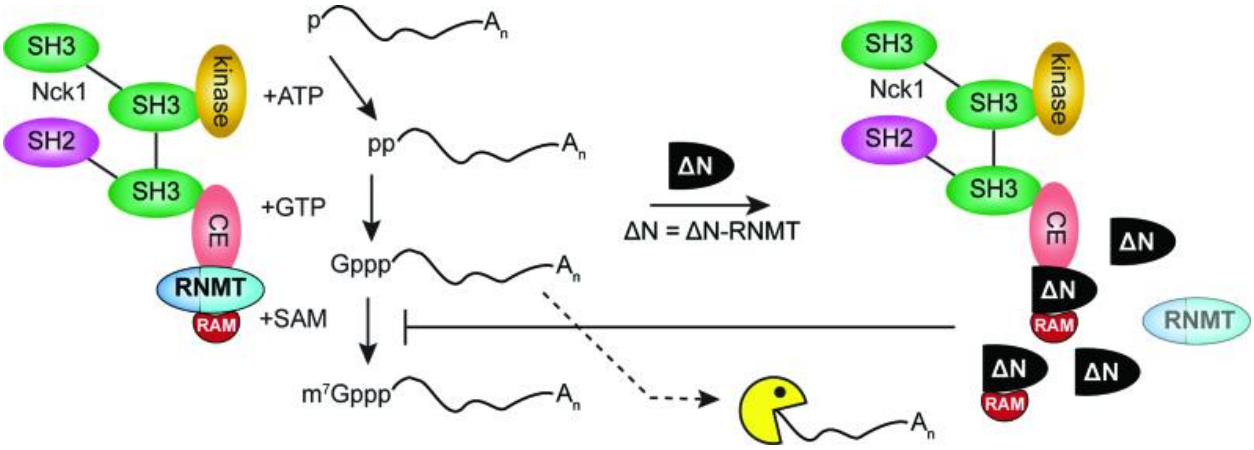

Figure 7: Schematic showing the interaction ΔN RNMT with the cytoplasmic capping complex. ΔN-RNMT sequesters away cytoplasmic capping complex machinery from wild-type RNMT. Cytoplasmic capping complexes that do not contain wild-type RNMT are incapable of methylating the re-constituted cap. An unmethylated cap is recognized by exonucleases in the cytoplasm, and these immature cap structures are degraded. (Source 10)

BioID validates novel protein interactors of cCE by western blot

A recent search for novel interactors (Jackson Trotman) of cCE using mass spectrometry (Bernice Agana) has elucidated several possible proteins that associate

with cCE and/or the cytoplasmic capping complex. This approach involved using a tagged form of cCE as a bait protein. After a 24 h expression of this bio-cCE plasmid, U2OS cells were gently crosslinked with formaldehyde to preserve protein-protein interactions and any RNA-protein interactions. Both the α and β isoforms of HSP90 were identified by this proteomics analysis as cCE-interacting proteins. HSP90 is a well-characterized, ATP-dependent protein chaperone that helps fold and stabilize a select but diverse group of client proteins. HSP90 functions as a chaperone constitutively, but its expression increases in response to protein-denaturing stressors to help stabilize client proteins (14).

I confirmed the *in vivo* interaction between cCE and Hsp90 using proximity-dependent biotinylation. HEK293 cells were transfected with either BirA*-cCE or BirA*-NES (figure 8A), and incubated in the presence of 1 µM biotin for 14 h. 24 h after transfection, cytoplasmic extracts were prepared and biotinylated were recovered on streptavidin beads. The preferential recovery of HSP90 from cells expressing BirA*-tagged cCE demonstrates that HSP90 indeed interacts with CE *in vivo* (figure 8B).

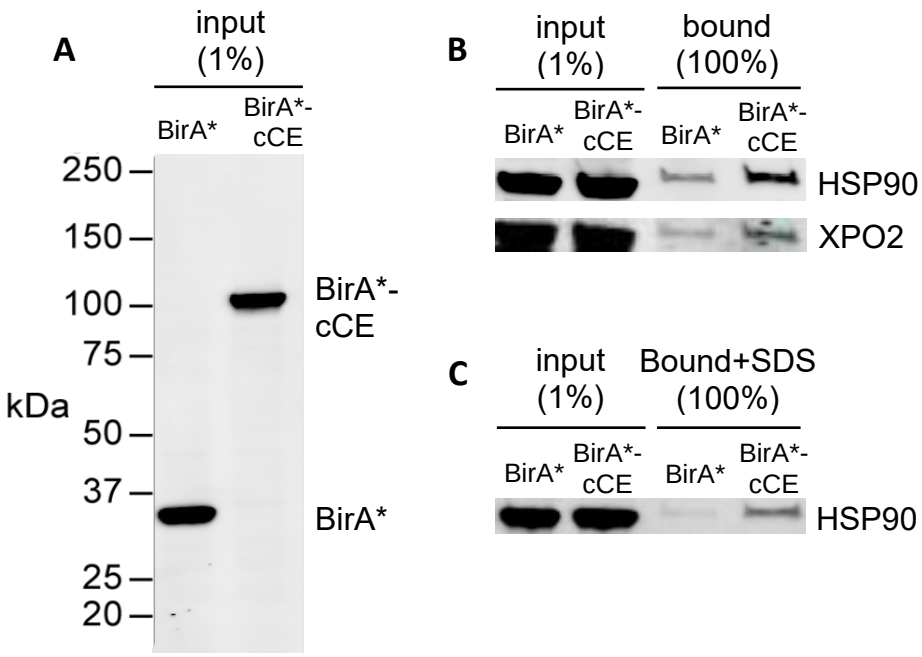

Figure 8: Validation of Hsp90 and Exportin 2 as protein interactors of cCE. A: Myc-BirA and Myc-BirA*-cCE were transiently transfected into HEK293 cells. Cytoplasmic extracts were prepared to measure the expression of each construct by western blot with an anti-Myc antibody. B: Proteins from the same extracts were recovered on streptavidin beads and analyzed by western blot with antibodies against Hsp90 and exportin-2. C: The experiment in (B) was repeated, except that 0.1% SDS was added to the Pulldown Buffer at the beginning of the streptavidin recovery (Source 11)*

The same method was used to validate the recovery of Exportin-2 (XPO2) (Figure 8B), a nuclear export receptor. Due to high background signal in pulldown samples from the negative control (BirA*-NES alone) seen for both HSP90 and XPO2, the pulldown using the same cytoplasmic extracts was repeated with 0.01% SDS to disrupt weaker, non-covalent protein-protein interactions. Proteins that were residually bound to streptavidin beads can then be more easily removed in subsequent washing steps. This lowers the level of off-target pulldown events but largely leaves the extremely tight association between biotin and streptavidin molecules intact. SDS pulldown has been used in

previous BioID studies (8). Introduction of SDS during streptavidin recovery significantly reduced the levels of Hsp90 recovered in the control samples while preserving the association between biotinylated Hsp90 and streptavidin in the BirA*-cCE sample (Figure 8C). Based on this result, SDS addition during streptavidin recovery was used during subsequent proteomic experiments to reduce the levels of recovered proteins in the control samples.

Mass spectrometry analysis of the cCE interactome using BioID

The main utility of BioID lies in the ability to identify novel interactors of a protein of interest using a mass spectrometry approach. To this end, U2OS cells were transfected with either pcDNA3.1/TO-myc-BirA*-NES or pcDNA3.1/TO-myc-BirA*-cCE. The backbone plasmid, pcDNA3.1, includes a neoR gene that confers resistance to the antibiotic Geneticin (G418). Cells with integrated plasmid DNA were selected for using 600 µg/mL G418. After about 2 weeks, individual colonies were isolated using cloning cylinders and grown to confluency as a clonal line. These cells were tested for their expression level of BirA* or BirA*-cCE using 1 µg/mL Doxycycline (data not shown). The BirA* control showed robust overexpression as expected, but the BirA*-cCE clones showed no detectable expression by western blot. To navigate this issue, a second G418 screen was conducted, and G418-resistant cells were pooled (individual clones were not isolated). This approach yielded a polyclonal mixture of BirA*-cCE expressing cells.

To recapitulate the most physiological cell conditions during proximity-dependent biotinylation, I decided to manipulate the expression level in stable cell lines by testing

various amounts of tetracycline-HCl, a doxycycline analog (figure 9). This approach allowed the expression of BirA*-cCE to mimic the physiological expression endogenous cCE. I first used an antibody against CE to directly compare BirA*-cCE to endogenous cCE expression from the same cytoplasmic extracts (Figure 9A). I decided that 0.2 µg/mL Tet-HCl best matched induced expression to endogenous expression. I then conducted a similar titration for the control line, BirA*. In order to directly compare BirA* to BirA*-cCE expression, I utilized a Myc tag present on the N-terminus of the BirA* protein in both the BirA* control and BirA*-cCE. I used an anti-myc antibody to visualize the expression of myc-BirA* and myc-BirA*-cCE on the same western blot. BirA* induction using 0.02 µg/mL Tet-HCl most closely matched the expression level of BirA*-cCE (Figure 9B). Using these levels of induction, immunofluorescence microscopy was used to characterize the subcellular localization of the tagged protein in each cell line. Both proteins seem to be nearly completely cytoplasmically localized (Figure 9C). About 90% of cells from the clonal BirA* line have a similar low level of expression (using anti-Myc primary antibody, stained red). The polyclonal BirA*-cCE line shows about 50% of cells expressing at a higher level, while other cells are unresponsive to induction, consistent with the observation that three isolated clonal cell lines did not express BirA*-cCE. These results are consistent with the western blot data in Figure 9B, in which the average expression of any given cell in the population is similar between the control and experimental lines. These two cell lines improve the scope of the BioID experiment by normalizing the expression of the BirA* between the control and experimental cell cultures.

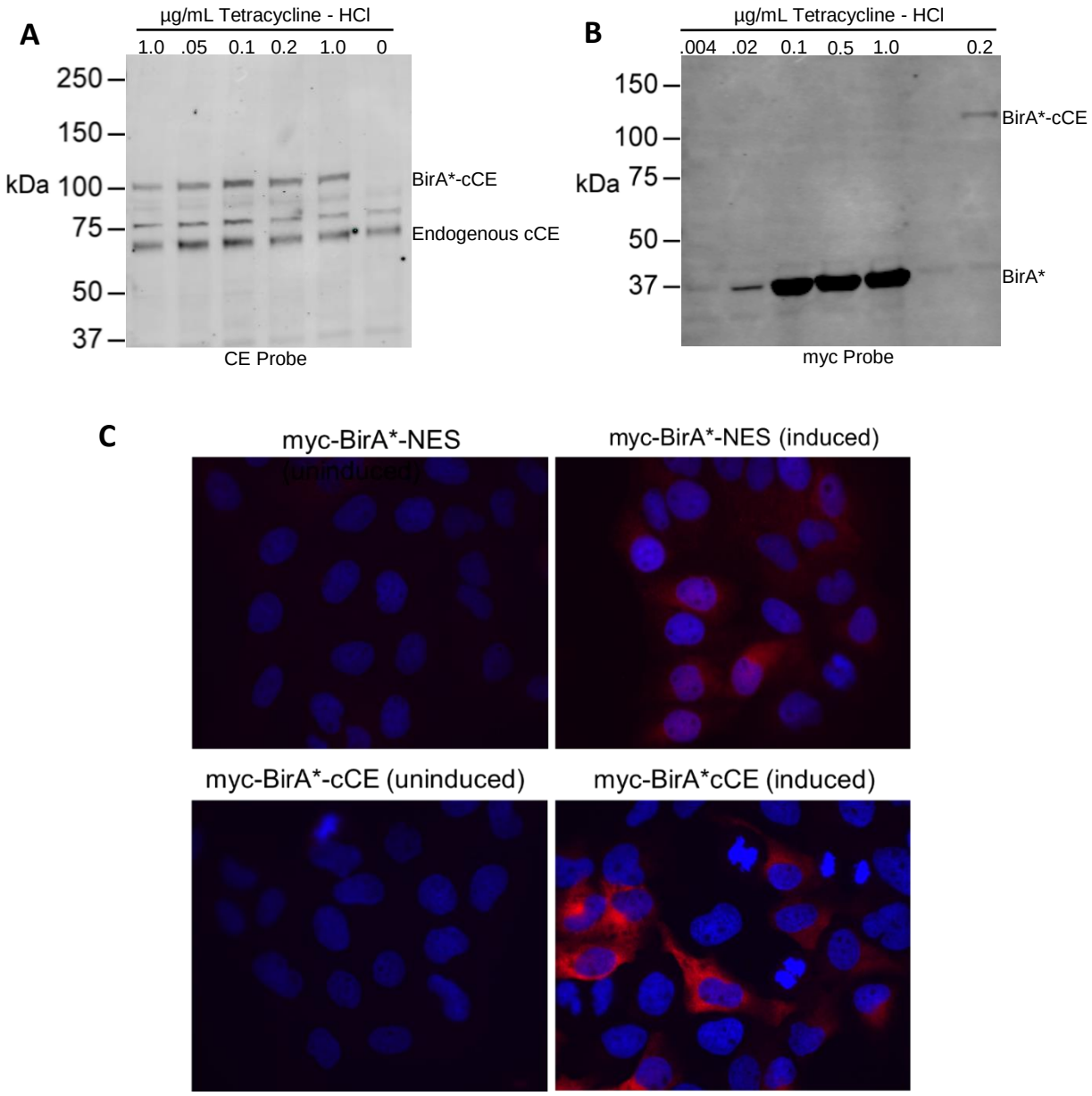

Figure 9: Characterization of stable UTOSTR cells used for proteomics. A: Cells transfected with BirA-cCE were selected for using G418. These cells were exposed to various amounts of Tet-HCl to determine their responsiveness to induction. The expression level of BirA*-cCE (induced) was compared to endogenous cCE expression using an antibody against CE. 0.2 µg/mL was chosen as it produced an expression level of BirA*-cCE most similar to that of endogenous cCE. B: Tetracycline induction (at various concentrations) comparing BirA* expression to 0.2 µg/mL Tet-HCl-induced BirA*-cCE expression. C: Expression pattern of BirA* and BirA*-cCE while induced with established concentrations of Tet-HCl.*

To prepare samples for mass spectrometry analysis, concentrations of Tet-HCl were used to induce expression of BirA* or BirA*-cCE at near-physiological level, as discussed above. Samples were prepared in triplicate. Six hours after induction, 10 µM biotin was directly added to the culture media. Twenty-four hours after induction, cells were harvested and streptavidin pulldowns were conducted using SDS-supplemented Pulldown Buffer as discussed above. Each pulldown reaction used 1.6 mg cytoplasmic protein quantified by Bradford assay. After washes, on-bead trypsin digestion was performed in preparation for liquid chromatography coupled to tandem mass-spectrometry (LC-MS/MS). MS/MS spectra were searched against a database containing tryptic peptide sequences of the human proteome, and the number of peptide spectral matches (PSMs) were tallied for each protein identified. Results from three independent experiments are presented in Table 1, with proteins sorted by enrichment in the BirA*-cCE samples over the BirA* samples. The analysis was filtered to exclude obvious peptide contaminants as well as BirA* and BirA*-cCE proteins themselves to allow for analysis of proteins with less sample representation. Surprisingly, no peptides identified for the known cCE-interacting proteins Nck1 or RNMT. The reason for this is unknown but could be caused by less-abundant peptide signals being overwhelmed by those from more abundant proteins during MS/MS analysis.

Because of the numerous peptide identifications enriched in the BirA*-cCE samples, it appears that cCE forms protein complexes beyond the previously described cytoplasmic capping complex. Many of these proteins are exclusively cytoplasmic,

suggesting that the cytoplasmic pool of CE has distinct function not present in the larger nuclear pool of CE.

Accession	Gene Symbol	Gene Name	cCE 1 PSMs	cCE 2 PSMs	cCE 3 PSMs	BirA 1 PSMs	BirA 2 PSMs	BirA 3 PSMs	cCE Enrichment Index
P35579	MYH9	Myosin-9	1	4	20	2			5.00
P49327	FASN	Fatty acid synthase	8	8	25	5	1	2	3.73
P50990	CCT8	T-complex protein 1 subunit theta	2	2	7	1			2.75
P13639	EEF2	Elongation factor 2			7				2.33
P26038	MSN	Moesin	8	2	11	4	2	1	2.10
P07437	TUBB	Tubulin beta chain	27	19	55	19	15	18	1.84
P30101	PDIA3	Protein disulfide-isomerase A3	5	9	8	8	1		1.83
Q9NRR2	TPSG1	Tryptase gamma			7	1			1.75
P49321	NASP	Nuclear autoantigenic sperm protein	2	8	4	3	2		1.75
P68371	TUBB4B	Tubulin beta-4B chain	22	17	49	18	13	17	1.73
P07355	ANXA2	Annexin A2	9	6	12	8	2	3	1.69
P29692	EEF1D	Elongation factor 1-delta	5	6	5	5	2		1.60
Q13085	ACACA	Acetyl-CoA carboxylase 1	49	48	66	44	15	41	1.58
P68363	TUBA1B	Tubulin alpha-1B chain	24	10	41	17	12	16	1.56
P11021	HSPA5	78 kDa glucose-regulated protein	10	24	11	21	5		1.55
P04792	HSPB1	Heat shock protein beta-1	6	8	7	7	3	1	1.50
P15311	EZR	Ezrin	9	2	14	9	3	2	1.47
P16949	STMN1	Stathmin	7	8	4	5	5		1.46
P06733	ENO1	Alpha-enolase	27	29	33	39	15	4	1.46
P06753	TPM3	Tropomyosin alpha-3 chain	2	7	1	3	1		1.43
P08238	HSP90AB1	Heat shock protein HSP 90-beta	18	5	22	8	8	13	1.41
P07195	LDHB	L-lactate dehydrogenase B chain	8	2	11	5	4	3	1.40
P07900	HSP90AA1	Heat shock protein HSP 90-alpha	12	3	18	7	5	9	1.38
P62937	PPIA	Peptidyl-prolyl cis-trans isomerase A	9	7	7	9	5		1.35
P43358	MAGEA4	Melanoma-associated antigen 4	1		3				1.33
P63313	TMSB10	Thymosin beta-10		4					1.33
Q09666	AHNAK	Neuroblast differentiation-associated protein AHNAK	98	194	124	156	70	84	1.33
P07237	P4HB	Protein disulfide-isomerase	13	18	10	20	5	3	1.32
P37802	TAGLN2	Transgelin-2	5	14	6	12	3	1	1.32
P14618	PKM	Pyruvate kinase PKM	14	5	20	12	10	5	1.30
P07737	PFN1	Profilin-1	4	1	5	4	1		1.25
P60174	TPI1	Triosephosphate isomerase	15	15	15	23	10		1.25

Table 1: Identification of human peptides interacting with cCE using mass spectrometry. Only peptides with an cCE enrichment score of 1.25 or greater are shown.

The mass spectrometry results garnered by BioID were then compared to the results from the pulldown of cCE following formaldehyde crosslinking (performed by Jackson Trotman) that was described above. Several of the BirA*-cCE highly-enriched PSMs identified by BioID were also identified as high-confidence PSMs by the crosslinking approach. These proteins are summarized in Table 2. Because many peptides were identified by both approaches, each method serves as a means of external validation for the other. This overlap suggests that enriched proteins are indeed real cCE interactors as opposed to an anomaly due to an uncontrolled aspect of the BioID experimental design.

Gene Symbol	Gene Name	XL-PD			BioID		
		Total cCE PSMs	Total EGFP PSMs	cCE Enrichment Index	Total cCE PSMs	Total BirA PSMs	cCE Enrichment Index
FASN	Fatty acid synthase	76	9	6.33	41	8	3.73
EEF2	Elongation factor 2	47	6	5.22	7	0	2.33
CCT8	T-complex protein 1 subunit theta	32	7	3.20	11	1	2.75
TUBA1B	Tubulin alpha-1B chain	70	19	3.18	75	45	1.56
TUBB	Tubulin beta chain	123	37	3.08	101	52	1.84
TUBB4B	Tubulin beta-4B chain	109	33	3.03	88	48	1.73
AHNAK	Neuroblast differentiation-associated protein AHNAK	16	3	2.67	416	310	1.33
HSP90AB1	Heat shock protein HSP 90-beta	85	29	2.66	45	29	1.41
HSP90AA1	Heat shock protein HSP 90-alpha	48	16	2.53	33	21	1.38
ENO1	Alpha-enolase	7	1	1.75	89	58	1.46
PFN1	Profilin-1	6	1	1.50	10	5	1.25
ANXA2	Annexin A2	20	12	1.33	27	13	1.69
HSPB1	Heat shock protein beta-1	8	3	1.33	21	11	1.50

Table 2: A summary of peptides that were identified by both the crosslinking-pulldown approach and BioID. Only peptides with a cCE enrichment score of 1.25 or greater are shown.

Discussion

The main aims of this project have been successfully explored. The BioID technology originally established in a perinuclear protein complex has been successfully altered to study cCE and its interactors. BirA*-cCE can be transiently transfected in HEK293 cells to overexpress the protein. Or, BirA*-cCE can be inducibly expressed at a moderate level in U2OS-TR cells to mimic the expression profile of endogenous cCE. The ability for the BioID technology to biotin-tag proteins *in vivo* provides a history of protein interactions over the course of an experimental time course. This novel approach provides a means of independent validation of other methods to study protein-proteins interactions and can dramatically strengthen the argument that two proteins associate *in vivo*. It can also expand the list of interacting factors compared to those identified by traditional pulldown experiments, because even transiently-interacting proteins have the potential to be biotin-tagged.

The utility of BioID to not only validate members of the core cytoplasmic capping complex but also to expand the cCE interactome has proven to a be a valuable tool to

explore the other potential functions of cCE. Based on the identification of proteins previously unknown to interact with capping enzyme, it is likely that the impact of cCE extends beyond a simple guanylyltransferase for a subset of cytoplasmic mRNAs. The exploration of a functional relationship of many proteins identified in Table 1 to cCE is a direction for future research. Many of the potential interactors have been shown to have RNA binding capability, but their impact on mRNA recapping specifically is not known. It is possible to some of these proteins are previously unknown factors that assemble with the core cytoplasmic capping complex on Nck1 and are necessary for formation of a stable cytoplasmic capping complex. Alternatively, these proteins may populate new protein complexes altogether, and cCE may be a common factor to multiple different cytoplasmic protein complexes.

Of the proteins identified, only Hsp90 has been explored in detail. Compounds that inhibit the chaperone function of Hsp90 can reduce the levels of both cytoplasmic and nuclear CE, suggesting that Hsp90 functions to stabilize cCE and reduce its turnover (11). Presumably, inhibition of Hsp90 also affects the recapped transcriptome, although this has yet to be established. As with Hsp90, many other proteins identified by both proteomic methods deserve further exploration to understand their functional relationship to cCE. One protein of immediate interest is fatty-acid synthase (FASN), because of its strong enrichment in both proteomics methods. FASN is a known RNA binding protein, and preliminary data suggests that functional Hsp90 is required to mediate the interaction between cCE and FASN. It is possible that the cCE-FASN interaction is necessary for effective cCE enzymatic activity. Alternatively, cCE may be contributing to efficient fatty-acid synthesis. Although the functional role of this

interaction is not yet characterized, it is becoming increasingly clear that cCE is contributing to cell processes other than its known role in cytoplasmic recapping.

There are also several proteins that are either themselves components of the cytoskeleton (multiple tubulins, myosin-9, stathmin) or involved in the attachment of the cytoskeleton to the plasma membrane (moesin, ezrin, annexin A2). cCE's potential role within the cytoskeleton is also unknown. One possibility is that cCE is mediating cytoskeletal changes that occur near the plasma membrane. Or, these cytoskeletal components could be recruit intact cytoplasmic recapping complexes to the cell periphery as a mechanism to regulate the cap status of transcripts on a local level. This recruitment would seemingly affect only a fraction of the total cCE pool, since cCE has been shown to be distributed throughout the entire cytoplasm including at the plasma membrane in several cell types (Schoenberg Lab, unpublished).

The current list of cCE interacting proteins is likely incomplete. There are several previously characterized cCE interactors that do not appear on my list, including Nck1 and RNMT. In addition to these known interactors, other potential complex members, like a 5' kinase and 2'O methyltransferase also do not appear. There are multiple explanations for these observations. The first is that these proteins are not sufficiently biotin-tagged under the specific experimental conditions used in mass spectrometry experiment. Because the molar concentration of proteins can vary over several orders of magnitude, it is likely that some interactors are incompatible for biotin-tagging due to low protein levels. In addition to these biological explanations, other issues likely arise during proteomic analysis. The ability for different peptides to be detected by the mass analyzer will vary and affect the success of identification of a mass to charge ratio. And,

at any given time, only the 15-most abundant peptides will be analyzed; it is possible that proteins that are bead-recovered at a low level will fall under the threshold of detection. It may be possible to intentionally exclude very abundant interactors during future mass spectrometry analyses, which would potentially allow for the detection of proteins recovered at a lower level. All of these issues are potential sources of error, but nonetheless, the current list of identified proteins provides several interesting routes for future research.

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